DISCOVERY OF A Ca^{2+}- AND CALMODULIN-DEPENDENT PROTEIN PHOSPHATASE

Probable identity with calcineurin ($CaM\text{-}BP_{80}$)

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1. Introduction

Incubation of phosphorylase kinase from rabbit skeletal muscle with cyclic AMP-dependent protein kinase and Mg-ATP causes a rapid phosphorylation of one serine residue on the β-subunit, followed by a phosphorylation of a further serine residue on the α-subunit [1–3]. The activation of phosphorylase kinase which accompanies this reaction is determined solely by the phosphorylation of the β-subunit [3–5]. Nevertheless, the serine residue on the α-subunit, as well as that on the β-subunit, becomes phosphorylated in vivo in response to adrenaline [6], suggesting that it may have a physiological function.

Several years ago we reported that extracts of rabbit skeletal muscle contained two different enzymes which dephosphorylated the β-subunit and α-subunit relatively specifically [7]. These enzymes were initially termed β-phosphorylase kinase phosphatase and α-phosphorylase kinase phosphatase [7], but were subsequently renamed protein phosphatase-1 and protein phosphatase-2 [3].

Protein phosphatase-2 which had been purified several hundred fold was reported to dephosphorylate histones H1 and H2b at similar rates to the α-subunit, and it also contained some glycogen synthase phosphatase and phosphorylase phosphatase activity [8]. Subsequently this enzyme was purified 2500-fold and shown to dephosphorylate a protein termed inhibitor-1 [9], which is a potent inhibitor of protein phosphatase-1 [3].

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Recent work in this laboratory has demonstrated that the 'protein phosphatase-2' preparations studied previously, contained varying amounts of two different enzymes that have been termed protein phosphatase-2A and protein phosphatase-2B [10]. Protein phosphatase-2A dephosphorylates the α-subunit of phosphorylase kinase 4–5-fold more rapidly than the β-subunit, but is a broad specificity enzyme that also dephosphorylates glycogen phosphorylase, glycogen synthase, inhibitor-1, histones H1 and H2B and other proteins ([10], T. S. I., P. C., unpublished). Protein phosphatase-2B dephosphorylates the α-subunit of phosphorylase kinase ~100-fold more rapidly than the β-subunit, and has a very restricted substrate specificity. It dephosphorylates inhibitor-1 and the α-subunit at comparable rates, but has no significant activity against glycogen phosphorylase, glycogen synthase, histones H1 and H2B or a number of other proteins (T. S. I., P. C., unpublished).

In the previous studies, the assays contained 1.0 mM Mn^{2+} since the dephosphorylation of the α-subunit of phosphorylase kinase was reported to require divalent cations [3,9]. Here, we have examined the metal ion requirement and structure of an 8000-fold purified preparation of protein phosphatase-2B which is free of contamination with protein phosphatase-2A. We demonstrate that Mn^{2+} can be replaced in the assays by μM levels of Ca^{2+}, and that the activity can be stimulated a further 10-fold by calmodulin. The subunit composition of the purified enzyme bears a remarkable resemblance to that of a major calmodulin-binding protein that has been isolated from bovine brain termed calcineurin [11] or $CaM\text{-}BP_{80}$ [12]. Further experiments have demonstrated that purified calcineurin contains a protein phosphatase activity

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with the same properties and specific activity as protein phosphatase-2B. This activity co-purifies with calcineurin through chromatofocusing experiments, suggesting that calcineurin and protein phosphatase-2B are the same protein.

2. Materials and methods

2.1. *Protein preparations*

Phosphorylase kinase [2], inhibitor-1 [13], inhibitor-2 [14] and calmodulin [15] were purified to homogeneity from rabbit skeletal muscle, and calcineurin [16] from bovine brain. Parvalbumin and actin from rabbit skeletal muscle were gifts from Dr Jacques Demaille, University of Montpellier and Dr Ian Trayer, University of Birmingham, respectively. ^{32}P-Labelled phosphorylase kinase and inhibitor-1 (10^8 cpm/μmol) were prepared by phosphorylation with cyclic AMP-dependent protein kinase as in [17].

Protein phosphatase-2B was isolated from extracts of rabbit skeletal muscle by a procedure which involved fractionation with ammonium sulphate, chromatography on DEAE–Sepharose, fractionation with polyethylene glycol, gel filtration on Sephadex G-200, chromatography on Affigel blue, and affinity chromatography on calmodulin–Sepharose. Protein phosphatase-2B bound to calmodulin–Sepharose in the presence of Ca^{2+} and could be eluted with EGTA. The enzyme was purified 8000-fold in an overall yield of 1%. The final specific activity measured in the presence of saturating concentrations of Ca^{2+} and calmodulin was 2.0 μmol phosphate released . min^{-1} . mg $protein^{-1}$ using inhibitor-1 as a substrate. The exact procedure will be detailed elsewhere (A. A. S., P. C., in preparation).

2.2. *Assay of protein phosphatase-2B*

Activity measurements were carried out at 30°C by measuring the release of $^{32}P_i$ from ^{32}P-labelled phosphorylase kinase (0.25 mg/ml or 0.8 μM in terms of $\alpha\beta\gamma\delta$ units) or inhibitor-1 (0.02 mg/ml or 1 μM). 0.05 ml of a solution containing 0.05 M Tris–HCl (pH 7.0 at 25°C), inhibitor-2 (50 U, see [14] for definition of units), bovine serum albumin (0.5 mg/ml), 0.1% (v/v) 2-mercaptoethanol, a Ca^{2+}–EGTA or Ca^{2+}–HEDTA [*N*8-(2-hydroxyethyl)-ethylenediamine-*N*,*N*′,*N*′-triacetate] buffer (see below), and protein phosphatase-2B, were warmed at 30°C for 2 min. The reaction was initiated with 0.01 ml ^{32}P-labelled substrate, and after 3 min the reactions were terminated and analysed as in [14]. Reaction rates were linear up to 15% phosphate released from each substrate, and the extent of dephosphorylation was therefore kept within this limit. Inhibitor-2 was included to inactivate any traces of protein phosphatase-1 that might be present in either purified protein phosphatase-2B or in the ^{32}P-labelled substrates. All of the ^{32}P-radioactivity released by protein phosphatase-2B could be extracted into acid–molybdate. This demonstrated that the ^{32}P-radioactivity released was P_i and not [^{32}P]phosphopeptides that might have been released by the action of proteinases.

2.3. *Calcium–EGTA and calcium–HEDTA buffers*

The concentration of the stock solution of $CaCl_2$ used to make up these buffers was determined by reference to a standard solution of Ca^{2+} (BDH Chemicals Ltd) using atomic absorption spectrophotometry. Free [Ca^{2+}] were computed using the following constants: Ca^{2+}–EGTA buffers; $pK_{H^+} = 9.46$ and 8.85; $pK_{Ca^{2+}} = 11.0$; Ca^{2+}–HEDTA buffers; $pk_{H^+} = 9.73$; $pK_{Ca^{2+}} = 8.14$. Ca^{2+}–EGTA buffers were used from 0.01–3 μM Ca^{2+} and Ca^{2+}-HEDTA buffers from 0.07–40 μM Ca^{2+}. The final concentrations of EGTA and HEDTA in the protein phosphatase assays were 5.0 mM and 1.0 mM, respectively.

3. Results and discussion

3.1. *Effect of Ca^{2+} and calmodulin on the activity of protein phosphatase-2B*

Protein phosphatase-2B had been shown to be dependent on Mn^{2+}. Since Mn^{2+} can replace Ca^{2+} in the activation of calmodulin-dependent enzymes, other divalent cations were tested for their ability to activate protein phosphatase-2B. These experiments demonstrated that μM levels of Ca^{2+} could indeed substitute for Mn^{2+}, while Mg^{2+} was ineffective. The dependence of activity on Ca^{2+} and calmodulin after the chromatography on Affigel blue is illustrated in fig.1. Half-maximal activation was observed at 1.0 μM Ca^{2+} in the absence and at 0.5 μM Ca^{2+} in the presence of 3×10^{-8} M calmodulin. Half-maximal activation by calmodulin occurred at 6×10^{-9} M, when assays were performed at 3 μM Ca^{2+}. Calmodulin increased the V_{max} of the reaction 10–20-fold and did not alter the K_m for inhibitor-1.

The dephosphorylation of the α-subunit of phos-

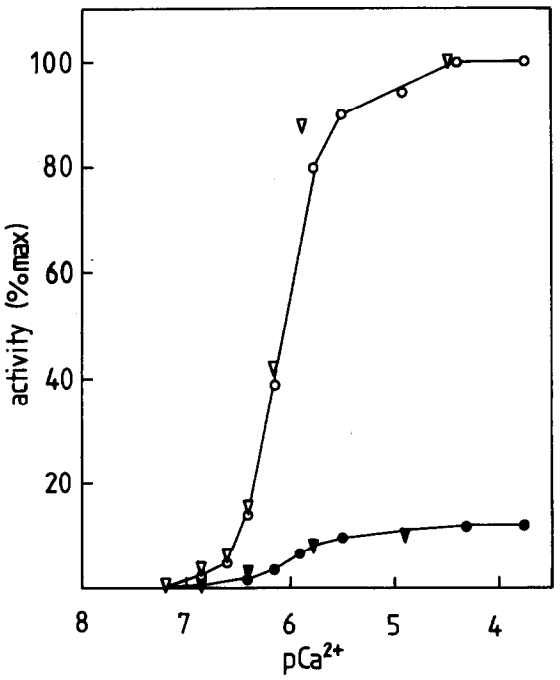

Fig.1. Dependence of protein phosphatase-2B on Ca^{2+} and calmodulin. Experiments were performed using inhibitor-1 as a substrate, and an enzyme preparation purified up to and including the chromatography on Affigel blue. The closed symbols show results obtained in the absence, and the open symbols results in the presence of 3×10^{-8} M calmodulin. Assays using Ca^{2+}–EGTA buffers are denoted by triangles and assays that employed Ca^{2+}-HEDTA buffers by circles.

phorylase kinase by protein phosphatase-2B was not stimulated by calmodulin. This is due to the presence of calmodulin as one of the subunits (the δ-subunit) of phosphorylase kinase [18,19]. The concentration of phosphorylase kinase (and therefore of calmodulin) in the assays was 2 μM or 100-fold higher than the $A_{0.5}$ for calmodulin. The ability of the δ-subunit to activate other calmodulin-dependent enzymes has been noted [18]. It is not yet clear whether the δ-subunit can activate protein phosphatase-2B and other calmodulin-dependent enzymes while it is still attached to phosphorylase kinase, or whether protein phosphatase-2B can extract some of the δ-subunit from the enzyme.

It should be noted that Mn^{2+} has a second and quite independent effect on the purified enzyme, in addition to its ability to replace Ca^{2+}. After the chromatography on calmodulin–Sepharose, protein phosphatase-2B was converted to a form that had a very low specific activity in the presence of Ca^{2+} and calmodulin. Reactivation could only be achieved by preincubation with Mn^{2+} (1.0 mM) and not with Ca^{2+}. Other properties were not altered however, e.g., stimulation by calmodulin and substrate specificity.

3.2. *Subunit structure of protein phosphatase-2B*

Protein phosphatase-2B showed one major protein staining band when examined by polyacrylamide gel electrophoresis in the absence of sodium dodecyl

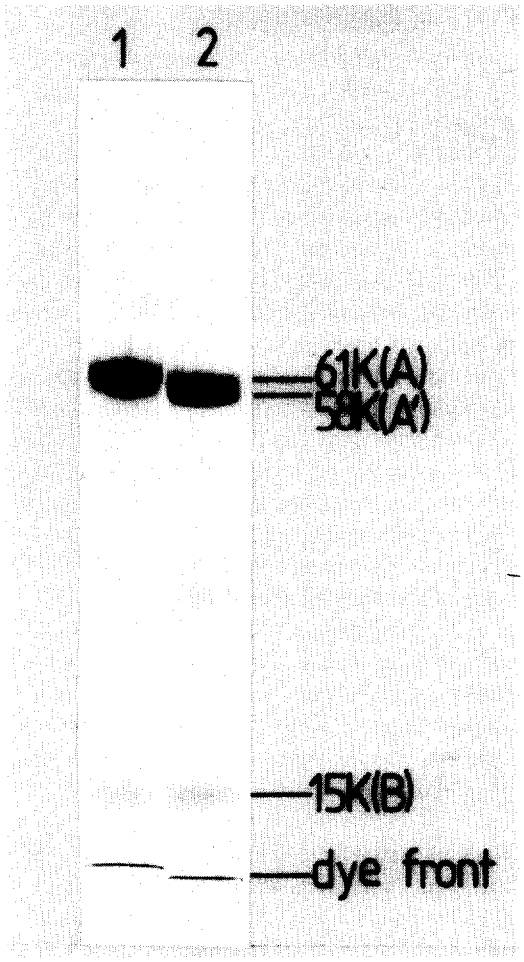

Fig.2. Electrophoresis of purified protein phosphatase-2B from rabbit skeletal muscle and calcineurin from bovine brain on $7\frac{1}{2}$% polyacrylamide gels run in the presence of sodium dodecyl sulphate: (1) calcineurin; (2) protein phosphatase-2B. The gels were stained with Coomassie blue and the migration is from top to bottom. The M_r-values were determined by calibration of the gels with the marker proteins phosphorylase *b* (M_r = 97 400), serum albumin (68 000), actin (42 000), carbonic anhydrase (29 500), calmodulin (16 700) and parvalbumin (11 900).

sulphate. When the gels were sliced and assayed, protein phosphatase-2B activity could only be detected at the position of the protein staining band (not shown).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate resolved the preparation into 3 major components A, A′ and B, having M_r-values of 61 000, 58 000 and 15 000, respectively (fig.2). Densitometric analysis of the gels gave a molar ratio (A + A′):B of 0.98:1.0, assuming uniform staining of each component with Coomassie blue. Component B migrated more rapidly than calmodulin (M_r = 16 700) and more slowly than parvalbumin (M_r = 11 900). The preparation also contained traces of 2 other components, M_r = 34 000 and M_r = 30 000 (fig.2).

The composition of protein phosphatase-2B was strikingly similar to that of a calmodulin binding protein isolated from brain, termed calcineurin [11] or CaM-BP$_{80}$ [12]. This protein is also composed of A and B subunits, M_r = 61 000 and 15 000, respectively, in a 1:1 molar ratio. These components co-migrated with subunits A and B of protein phosphatase-2B (fig.2). The app. M_r of protein phosphatase-2B determined by calibration of a Sephadex G-200 column (98 000 ± 4000) was very similar to that of calcineurin [11].

Purified preparations of calcineurin from brain contained a protein phosphatase activity that was indistinguishable from protein phosphatase-2B. It dephosphorylated the α-subunit of phosphorylase kinase 100-fold faster than the β-subunit, had the same relative activity towards phosphorylase kinase and inhibitor-1 and did not dephosphorylate phosphorylase *a*. Its activity was Ca^{2+}-dependent and stimulated 10-fold by calmodulin. Fresh preparations of calcineurin had a specific activity which was almost identical to that of purified protein phosphatase-2B from skeletal muscle. Protein phosphatase-2B from brain was also found to co-purify with calcineurin when a fraction containing a mixture of partially purified calmodulin-binding proteins was subjected to chromatofocusing (fig.3). These experiments strongly suggest that calcineurin and protein phosphatase-2B are the same protein, although the possibility that protein phosphatase-2B is a trace contaminant in calcineurin preparations is still not entirely excluded.

The relationship between the A and A′ components of protein phosphatase-2B remains to be established, but several possibilities are the following. The

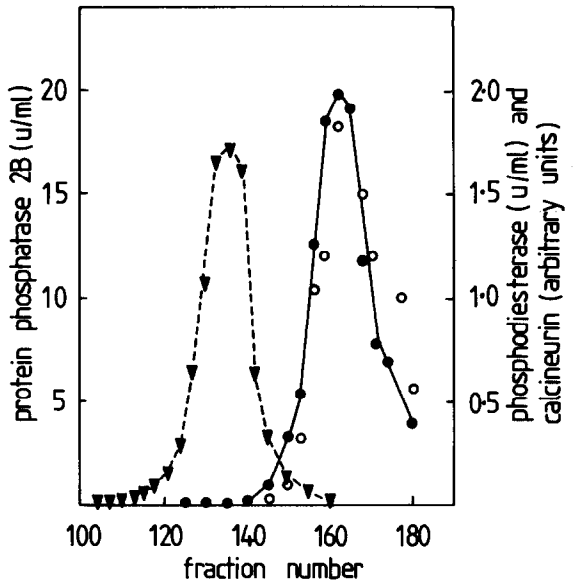

Fig.3. Chromatofocusing of calmodulin-binding proteins from brain. Bovine brain calmodulin binding proteins were prepared by a modification of the procedure in [21] (fraction IV). A 15 ml aliquot (A_{280} = 1.86) was applied to a 16.5 × 2.5 cm column of Sephadex G-10 equilibrated in 0.025 M imidazole–HCl (pH 7.4). The UV absorbing material with a conductivity <2.7 mS (0°) was pooled and immediately applied to a 30 × 0.9 cm column of polybuffer exchanger 94 (PBE-94) equilibrated in 0.025 M imidazole–HCl (pH 7.4) according to the manual supplied by Pharmacia Fine Chemicals. The protein was eluted with 250 ml polybuffer 74–HCl (pH 4.0). The flow rate was 20 ml/h and the fraction size 1.6 ml. At the end of the run, the pH of individual fractions was adjusted to 7.5 at 0°C by addition of 0.1 vol. 1.0 M Tris–HCl (pH 8.0). Cyclic AMP-phosphodiesterase (▼---▼) was assayed in the presence of Ca^{2+} and calmodulin (10^{-6} M) as in [21] and protein phosphatase-2B (●——●) in the presence of 1.0 mM Mn^{2+} using phosphorylase kinase (0.8 μM) as a substrate. Aliquots (50 μl) were electrophoresed on 7–15% gradients of polyacrylamide in the presence of SDS [16]. Calcineurin (○——○) was estimated by densitometric analysis of the gel patterns using both the 15 000 and the 61 000 M_r polypeptides and the levels are expressed in arbitrary units. The overall recovery of protein was 50%, phosphodiesterase 25%, calcineurin 30% and protein phosphatase-2B 10%. The material applied to the chromatofocusing column contained 6 major protein staining bands, M_r = 230 000, 150 000, 140 000, 61 000 (calcineurin A), 50 000 and 15 000 (calcineurin B). A minor component (M_r = 58 000) was cyclic AMP-phosphodiesterase. The M_r = 230 000, 150 000 and 140 000 species were present in the flowthrough fractions (20–25), and the fractions eluting between phosphodiesterase and calcineurin contained the same 3 components as well as the M_r = 50 000 component. Phosphodiesterase was eluted at pH 4.7 and protein phosphatase-2B and calcineurin at pH 4.1. The first 100 fractions (not shown) contained no detectable protein phosphatase-2B or calcineurin.

A′ component could be derived from the A component by limited proteolysis. The A component could be a phosphorylated or otherwise modified form of the A′ component. The A and A′ components could represent different isoenzymes of protein phosphatase-2B.

It has been established that component A of calcineurin is the subunit which binds to calmodulin, while component B is a calcium-binding protein that binds 4 Ca^{2+} with affinities in the μM range [11]. Since protein phosphatase-2B is a Ca^{2+}-dependent enzyme that can be activated a further 10-fold by calmodulin (fig.1), it is attractive to speculate that component A is the catalytic subunit and that its dependence on Ca^{2+} in the absence of calmodulin is conferred by component B. If these ideas are correct, then protein phosphatase-2B would resemble phosphorylase kinase [19,20] in being regulated by 2 different calcium binding proteins one of which is an integral subunit of the enzyme, the other only interacting in the presence of Ca^{2+}.

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